Photochemical regeneration of NADPH using the enzyme ferredoxin-$NADP^+$ reductase

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Ferredoxin-$NADP^+$ reductase and the electron transfer protein ferredoxin (or flavodoxin) were used to photochemically regenerate NADPH. The presence of ferredoxin enhanced the rate of NADPH production almost fivefold when either proflavin or 5-deazariboflavin was used as photosensitizer. The very high turnover numbers (8,100 min^{-1}) obtained for the enzyme in this system indicate the efficiency of the process. The system was also shown to be very stable, since total turnover numbers of approximately 3.2 × 10^5 were obtained during continuous operation.

Keywords: Photoproduction; NADPH; FNR; ferredoxin

Introduction

The reduced pyridine nucleotides NADH and NADPH play an essential role as cofactors of many enzymatic reactions. Many important chemical and pharmaceutical compounds can be synthesized through enzymatic reactions in which the reduced cofactors should be present in stoichiometric amounts. Due to the very high cost of these compounds, several methods have been designed for the regeneration of the reduced nucleotides at an industrial level.[1-3]

The simplest procedures described to produce large amounts of reduced pyridine nucleotides require the use of crude or purified preparations of the appropriate enzymes and the corresponding substrates.[4,5] This is very costly, since both the enzyme and the substrate are expensive. Moreover, the use of batch-type reactions adds some complications, since equilibrium is usually reached when only part of the reaction has taken place and also because the enzyme preparation cannot be used for further cycles. This problem can be overcome by the *in situ* regeneration of the reduced cofactor with the use of a sacrificial substrate as the ultimate source of reducing power, or by means of electrochemistry.

Several enzyme systems have been described for the photochemical or electrochemical reduction of a mediator that is able to transfer electrons to the enzyme at sufficiently high rates. They all present, nevertheless, the drawback of very low turnover numbers for the catalyst as well as the low stability of the enzyme preparation in the bioreactor conditions. The reaction would proceed at sufficiently high rates only if the overall process, i.e. the interaction of the mediator with the photosensitizer or the electrode and with the enzyme, takes place very efficiently. This requires the mediator to be well suited both thermodynamically (sufficiently negative redox potentials) as well as kinetically (efficient interaction with the active center of the enzyme).

Ferredoxin-$NADP^+$ reductase is the enzyme involved in the transfer of electrons generated during the photosynthetic process from ferredoxin to $NADP^+$. It has been used in both photochemical and electrochemical systems to reduce $NADP^+$. One system uses the cathode to reduce methyl viologen, which transfers electrons to the enzyme FNR.[6] In a later work the regeneration cycle of NADPH was used for organic synthesis by coupling enzymes that use the reduced pyridine nucleotide.[7] The photochemical method uses the water-soluble photosensitizer Ru(bpy)[8] or the semiconductors CdS or TiO_2[9] to reduce methyl viologen, which then reduces the reductase. Direct reduction of FNR by methyl viologen is a relatively slow process due to limited accessibility to the solvent of the prosthetic group FAD in the enzyme. Flavodoxin is a small-sized, FMN-containing flavoprotein that is present in microorganisms usually in conditions of iron deficiency

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and replaces ferredoxin in all the reactions in which this iron protein participates.[10]

We present data on the continuous photochemical reduction of $NADP^+$ by FNR in the presence of the electron transport protein ferredoxin (or flavodoxin), which acts as a partner in the physiological reaction catalysed by this enzyme. Substantially higher rates of conversion are obtained in comparison with previous systems. At the same time it is shown that the system is stable for periods of up to 30 h of continuous operation.

Materials and methods

Ferredoxin-$NADP^+$ reductase and flavodoxin were purified from *Anabaena* PCC 7119 as described previously,[11,12] from cells that were grown autotrophically on nitrate and under low iron concentration (2.6 μM) to allow synthesis of flavodoxin. Under these conditions, significant amounts of ferredoxin are also produced, which was purified by chromatography in DEAE–cellulose and gel filtration in Sephadex G-50 after treatment with DNAase and RNAase to remove the contaminant nucleic acids.

5-Deazaribloflavin was a kind gift from Dr. D. E. Edmondson of Emory University, Atlanta. 1,1'-Propylene-2,2'-bipyridylium was donated by Dr. G. Tollin from the University of Arizona. All other reagents were purchased from commercial sources and were of analytical grade.

Steady-state experiments were carried out at room temperature in a 1.5-ml necked cuvette containing 1 ml reaction mixtures. Anaerobic conditions were established by repeated cycles of evacuation and flushing with argon, which had been purified by passage over a heated BASF catalyst.

NADPH was produced continuously using a similar apparatus to that described by Butterworth *et al.*[13] in an Amicon 10-ml ultrafiltration cell with a YM-5 membrane. Reagents contained in a reservoir entered the cell under nitrogen pressure. Illumination of 10,000 lux was achieved with a 50 W halogen lamp fitted with an Oriel Corp. blue filter number 59855, with a cutoff band between 320 and 550 nm. The amount of NADPH formed was measured by the increase in absorbance at 340 nm.

Table 1 Characterization of the ferredoxin-dependent reduction of $NADP^+$ by *Anabaena* PCC 7119 ferredoxin-$NADP^+$ reductase with a 5-deazariboflavin photosystem

System	NADPH formed (nmol min^{-1})
Complete	28.5
Minus 5-deazariboflavin	0
Minus ferredoxin	6.2
Minus FNR	1.2
Minus EDTA	4.7
Minus $NADP^+$	0
Complete, in the dark	0
Complete, aerobic without shaking	16.8

The complete system included (in a final volume of 1 ml) 60 μM 5-deazariboflavin, 7.5 μM ferredoxin, 25 nM FNR, 20 mM EDTA, 2 mM $NADP^+$ in 20 mM Tris/HCl pH 8. The reaction was carried out in anaerobic conditions in blue light at 10,000 lux

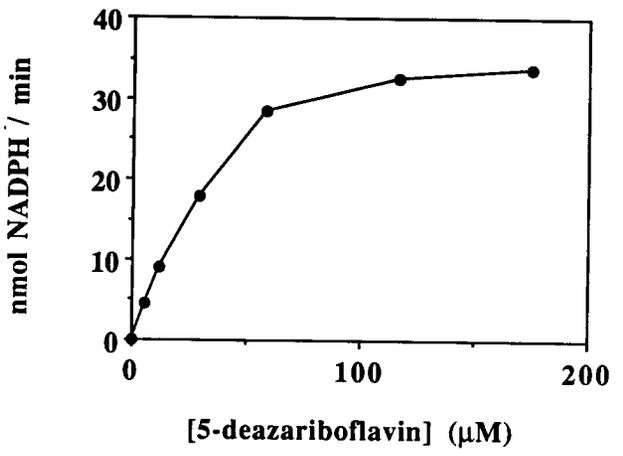

Figure 1 5-Deazariboflavin concentration dependence of the photoreduction of $NADP^+$. The reaction mixture contained (in a volume of 1 ml) 25 nM FNR, 7.5 μM ferredoxin, 20 mM EDTA, 2 mM $NADP^+$, and 5-deazariboflavin in 20 mM Tris/HCl pH 8

Results

Irradiation of a solution containing 5-deazariboflavin in the presence of EDTA produced the reduction of $NADP^+$ catalysed by the enzyme FNR. *Table 1* shows that photosensitizer, enzyme, light, and $NADP^+$ are absolutely essential for the reaction to take place. It is also shown that in the absence of ferredoxin the rate of reduction of $NADP^+$ is approximately 20% of that obtained with the complete system.

The rate of $NADP^+$ reduction decreases significantly under aerobic conditions, since oxygen competes efficiently with the enzyme in the reoxidation of reduced flavins. Continuous irradiation of an unshaken sample provided essentially anaerobic conditions, because diffusion of oxygen into the reaction mixture was avoided by the immediate reaction with the reduced flavin at the sample surface. The residual activity observed in the absence of EDTA indicates that the Tris–HCl buffer, which is present in the solution, acts as a sacrificial substrate in the photochemical reaction.

Figure 1 shows that the concentration of the photosensitizer required to saturate the system is of the order of 100 μM. All the experiments were performed at 60 μM 5-deazariboflavin, where approximately 80% of the maximal rate is obtained.

The capacity of several flavin compounds to act as photosensitizers of the FNR-dependent $NADP^+$ reduction was studied. *Table 2* shows that proflavin, which has a very negative redox potential (−730 mV), gives only 25% of the rate with 5-deazariboflavin. Other flavin compounds, such as riboflavin, FMN, and FAD, are not able to reduce the enzyme, since their redox

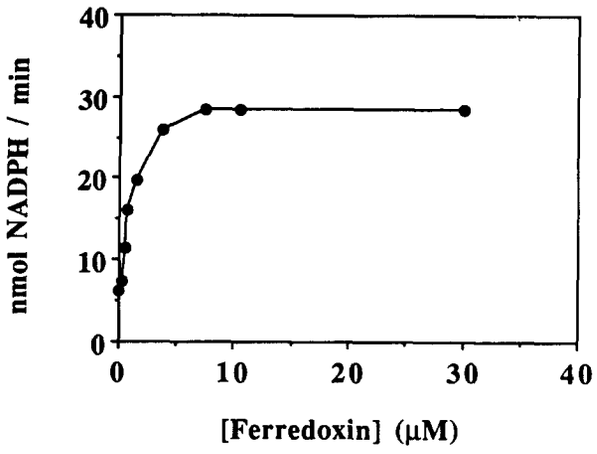

Figure 2 Ferredoxin concentration dependence of the photoreduction of $NADP^+$. The reaction mixture contained (in a volume of 1 ml) 25 nM FNR, 60 μM 5-deazariboflavin, 20 mM EDTA, 2 mM $NADP^+$, and ferredoxin in 20 mM Tris/HCl pH 8

potentials are about −200 mV compared to −320 mV for FNR.[11]

The effect of the concentration of ferredoxin on the photochemical reaction was studied and the results are presented in *Figure 2*, where the sharp increase in the rate of $NADP^+$ photoreduction with the concentration of the electron transfer protein is shown. Ferredoxin is the natural electron donor to FNR and acts by shuttling electrons between the light-reduced photosynthetic membranes and the enzyme, which is located outside the stromal cavities. Its role in the *in vitro* system could also be to transfer electrons from the reduced flavin, which has a very negative redox potential, to the enzyme. It is known that ferredoxin forms very tight complexes with FNR (K_d = 3.7 μM) which are functional during the photosynthetic electron transfer in which this enzyme participates.[11] Flavodoxin, which replaces ferredoxin in forming functional complexes which are similarly stable (K_d = 6.4 μM, ref. 14) produces almost the same increase in the activity, but at concentrations slightly higher than ferredoxin (see *Table 3*). At a concentration of around 200 μM, methyl viologen and the analog 1,1'-propylene-2,2'-bipyridyl, which can be easily reduced by flavins,[15] showed $NADP^+$ reduction rates of about 60% of that obtained with ferredoxin.

It was also observed that the pH of the solution had very little effect on the rate of NADPH production, since similar values were obtained at pH values from 7 to 10, with a maximum at pH 8 (not shown). Only at pH 6 was the rate significantly lower, reaching approximately 65% of the maximal rate.

The time course for the photochemical reduction of $NADP^+$ in the presence of the enzyme FNR is linear during the first 10-min period (*Figure 3*), showing a turnover number of 8,100 min^{-1}. This high rate declines slowly as the reaction progresses, reaching practically zero after 8–10 h of continuous illumination. Operation of this system by the batch method implies that only 8–10% of the total $NADP^+$ present in the solution has been reduced after this illumination period, the limiting factor being the photodegradation of the sensitizer. The enzyme system is stable after this period, as will be shown later.

Once the system had been characterized, the continuous production of NADPH was undertaken. The proteins were immobilized in an ultrafiltration cell with a membrane that allowed the passage of molecules of molecular weights below 5 kDa. For this continuous system, some of the components of the reaction mixture were changed; ferredoxin was replaced by flavodoxin, since the iron protein is much more unstable than the flavoprotein and also because it binds very tightly to the ultrafiltration membrane. 5-Deazariboflavin was also replaced by proflavin because this acridine compound is much more economical and more readily available than 5-deazariboflavin.

Table 2 Efficiency of different flavin photosystems on the reduction of $NADP^+$

Flavin	Concentration (μM)	NADPH formed (nmol min^{-1})
5-Deazariboflavin	30	17.9
	60	28.5
	120	32.6
	175	33.7
Riboflavin	10	0.3
	100	0.8
Proflavin	10	5.9
	25	8.1
	50	8.6

Experimental conditions were as for the complete system in Table 1. Where indicated, riboflavin or proflavin at the indicated concentrations replaced 5-deazariboflavin

Table 3 Efficiency of different electron carriers in the photoreduction of $NADP^+$

Electron carrier	Concentration (μM)	NADPH formed (nmol min^{-1})
Ferredoxin	7.5	7.1
	15	7.0
Flavodoxin	15	5.1
	30	6.7
	45	6.8
Methyl viologen	200	3.6
	400	3.9
1,1'-Propylene-2,2'-bipyridylium	200	4.8
	400	5.1

The complete system included (in a final volume of 1 ml) 60 μM 5-deazariboflavin, 0.92 nM FNR, 20 mM EDTA, 2 mM $NADP^+$, and electron carrier at the indicated concentration in 20 mM Tris/HCl pH 8. *The reaction was carried out in anaerobic conditions in* blue light of 10,000 lux

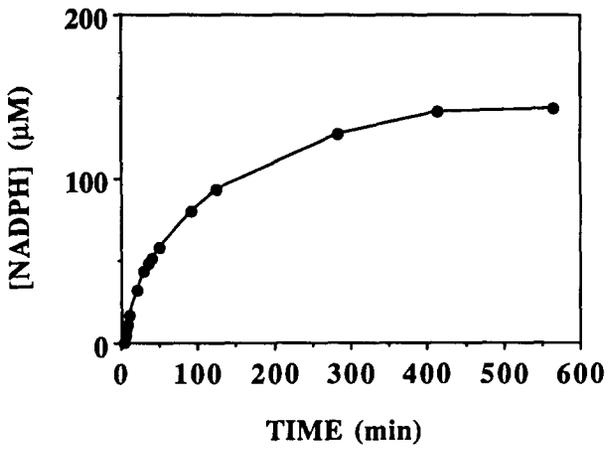

Figure 3 Time course of batch $NADP^+$ photoreduction. The system contained (in a volume of 1 ml) 60 μM 5-deazariboflavin, 0.46 nM FNR, 7.5 μM ferredoxin, 20 mM EDTA, and 2 mM $NADP^+$ in 20 mM Tris/HCl pH 8

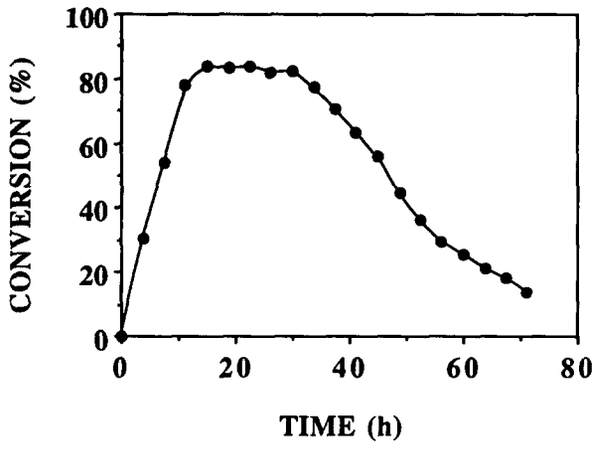

Figure 4 Time course of continuous $NADP^+$ photoreduction. The reactants mixture contained 80 μM proflavin, 0.1 mM $NADP^+$, and 20 mM EDTA. The bioreactor had a volume of 10 ml and contained 150 nM FNR and 60 μM flavodoxin. Residence time was 200 min

Figure 4 shows the time course of the reaction with the percentage of $NADP^+$ conversion. It can be observed that it takes a long time to reach the optimal rate of conversion, which is slightly over 80% of the total $NADP^+$ present in the solution. The system is stable at the maximal rate for about 20 h, after which it declines. The rate of conversion of about 80% was obtained when the solution was passing through the cell at a flow rate of 3 ml h^{-1}. This means a residence time for the $NADP^+$ in solution of approximately 200 min. At higher flow rates the conversion obtained was lower: 30% for a residence time of 50 min. These times are much longer than those required when the reaction is carried out in an anaerobic cuvette, since the oxygen present in the cell is competing with the proteins for the reduced flavin.

Discussion

Many enzyme-catalysed reactions that use NADH and NADPH are of potential interest for the synthesis of organic compounds, but they have the disadvantage of requiring the reduced cofactors, which are very expensive and difficult to obtain. This is even more important in the case of NADPH because there are not many enzymes capable of reducing $NADP^+$. Glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase have been used, at the laboratory level, to regenerate NADPH, but their industrial application is very limited due mostly to the very costly substrates they use. The cyanobacterium *Anabaena* produces large amounts of ferredoxin-$NADP^+$ reductase, which can be isolated and purified, together with flavodoxin, in a few steps.[16] The availability of clones of both proteins FNR and flavodoxin,[17,18] which can be expressed in *E. coli*, will enable large amounts of both proteins to be isolated much more easily, while also allowing mutations to be introduced in positions that produce a positive effect on the stability of both proteins.

The system we describe here has the advantage, over other electrochemical and photochemical methods described,[7,8] of showing higher activities, and also of being quite stable during operation. The very high catalytic activity (TN = 8,100 min^{-1}) observed during operation in the batch system is higher than the turnover number described for the enzyme in other reactions where it can be assayed. In all cases the TN is lower: 5,200 min^{-1} for the photochemical reaction in which $NADP^+$ is reduced by electrons donated by illuminated photosynthetic membranes; similarly low values are obtained when the enzyme is assayed in the opposite direction, i.e. using NADPH as the reductant, in which case turnover numbers of around 6,000 min^{-1} have been described.[11,14] Total turnover numbers (i.e. the total number of cycles that the enzyme undergoes in order to produce the indicated concentration of NADPH) in the range of 3.2×10^5 are obtained during batch operation of the system.

The photochemical system we describe here has the disadvantage of being more complex than other systems, since an additional protein is required to be present in the reactor for optimal activity. The easy availability of flavodoxin, which can be obtained at the laboratory level in gram quantities, together with the this protein's high stability, makes this method very promising for the reduction of economically interesting compounds. Coupling of this system to other NADPH-requiring enzymes would allow the synthesis of compounds of commercial interest such as steroid hormones or other pharmaceuticals.

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